

# Dynamics of Bacteriophage Genome Ejection *In Vitro* and *In Vivo*

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**Abstract.** Bacteriophages, phages for short, are viruses of bacteria. The majority of phages contain a double-stranded DNA genome packaged in a capsid at a density of  $\sim 500$  mg/ml. This high density requires substantial compression of the normal B form helix, leading to the conjecture that DNA in mature phage virions is under significant pressure, and that pressure is used to eject the DNA during infection. A large number of theoretical, computer simulation and *in vitro* experimental studies surrounding this conjecture has revealed many — though often isolated and/or contradictory — aspects of packaged DNA. This prompts us to present a unified view of the statistical physics and thermodynamics of DNA packaged in phage capsids. We argue that the DNA in a mature phage is in a (meta)stable state, wherein electrostatic self-repulsion is balanced by curvature stress due to confinement in the capsid. We show that in addition to the osmotic pressure associated with the packaged DNA and its counterions, there are four different pressures within the capsid: pressure on the DNA, hydrostatic pressure, the pressure experienced by the capsid, and the pressure associated with the chemical potential of DNA ejection. Significantly, we analyze the mechanism of force transmission in the packaged DNA, and demonstrate that the pressure on DNA is not important for ejection. We derive equations showing a strong hydrostatic pressure difference across the capsid shell. We propose that when a phage is triggered to eject by interaction with its receptor *in vitro*, the (thermodynamic) incentive of water molecules to enter the phage capsid flushes the DNA out of the capsid. *In vivo*, the difference between the osmotic pressures in the bacterial cell cytoplasm and the culture medium similarly results in a water flow that drags the DNA out of the capsid and into the bacterial cell.

## 1. Introduction

Bacteriophages, or phages, are viruses of bacteria. Phages consist of a protein capsid that encapsidates their genome, and a tail — a hollow tube connected to the capsid via a portal complex. During infection, the phage tail attaches to a host bacterium, punctures the cytoplasmic membrane and its genome translocates through the portal and the tail into the bacterial cytoplasm. Infection initiates the phage life-cycle: within the bacterial cytoplasm the genome is transcribed and replicated, phage proteins are synthesized, and new genome copies are packaged into newly assembled capsids. The cycle ends with lysis of the host cell and the release of multiple progeny. Understanding the mechanism(s) of phage genome ejection is important, not only in the insights it provides on DNA structure but also to provide a model for how eukaryotic viruses may release their nucleic acid into the cytoplasm or nucleus of an infected cell.

The genome of a mature phage virion is usually a B-form, double-stranded DNA (dsDNA). A common phage may have 35-50 kb DNA (DNA and genome will be used interchangeably) packed into a capsid of  $\sim 60$  nm diameter, i.e., the phage DNA is packaged tightly within the capsid in a condensed state, at a linear compression factor  $\sim 250$ , or at a density  $\sim 500$  mg/ml [1–14, 20, 21]. More than five decades ago, this compression led to the conjecture (henceforth referred to as the “*pressure-conjecture*”) that DNA in a mature phage capsid is under significant pressure, and is kept in place by means of a “plug”: protein(s) in the tail tube. When the plug is opened by the action of the appropriate receptor, the pressure of the DNA causes its release into the cytoplasm of an infected cell in a biologically passive manner [22–25]. The difficulty of obtaining experimental kinetic data for the ejection of any phage genome into an infected cell resulted in the conjecture becoming “fact”. The few published examples that were inconsistent with the theory elicited little response and most textbooks simply refer to the “DNA injection” step without further elaboration. However, at the beginning of this millennium, the pressure conjecture resurfaced in the biophysics community. A single molecule study reported that the packaging motor of phage  $\phi 29$  is capable of packaging DNA into a phage capsid against a force of magnitude  $\geq 60$  pN [26]. The force, which was assumed to be totally conserved, and thus available for the subsequent ejection step, and which therefore can be termed as the “ejection force”, was simply defined as the pressure on the DNA within the capsid, multiplied by the cross-sectional area of the unit cell corresponding to the hexagonal lattice arrangement of the packaged DNA. It was estimated that the pressure on the packaged DNA, as well as the pressure on the inside of the capsid is in the order of 60 atm for a mature phage virion. This work was quickly followed by a large number of computer simulations [27–30], theoretical analyses [31–35], and *in vitro* experimental studies [36–41, 44–48] surrounding the pressure-conjecture, a significant fraction of these studies stating the 60 atm pressure on the packaged DNA for a mature virion as a matter of fact.

Many recent theoretical, computer simulation and *in vitro* experimental studies have been principally directed to quantify how the pressure on the DNA can be

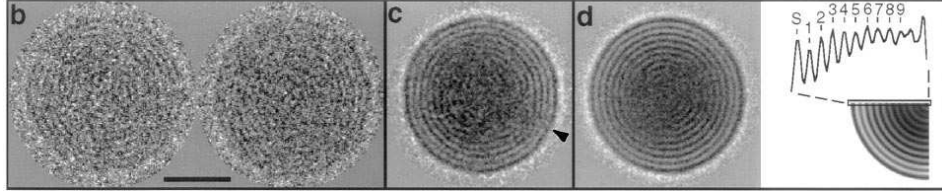
understood from a thermodynamic perspective. However, to the best of our knowledge, the issue of the mechanical transmission of the pressure along the packaged DNA helix has received no attention. Putting aside that question for the moment, a general consensus has been reached on a thermodynamic description of the pressure on the DNA. DNA is a charged polymer with persistence length  $\simeq 50$  nm; DNA confined inside a capsid of diameter  $\sim 50$  nm has a large free energy cost  $F$ , relative to the state of the DNA outside the capsid. The pressure on the packaged DNA can be thermodynamically derived from this free energy. However, fundamental disagreements remain between different theories on the thermodynamic origin of the pressure acting on the packaged DNA. In light of these disagreements, together with the question of how mechanical transmission of force along a flexible polymer, naturally raises the question: “Does the pressure-conjecture necessitate a biologically passive ejection force”?

In order to appreciate the outstanding issues more easily, we provide a brief overview of the structure of packaged phage DNA, and existing approaches to describe phage DNA packaging and ejection.

### *1.1. Structure of the packaged DNA in mature phage virions*

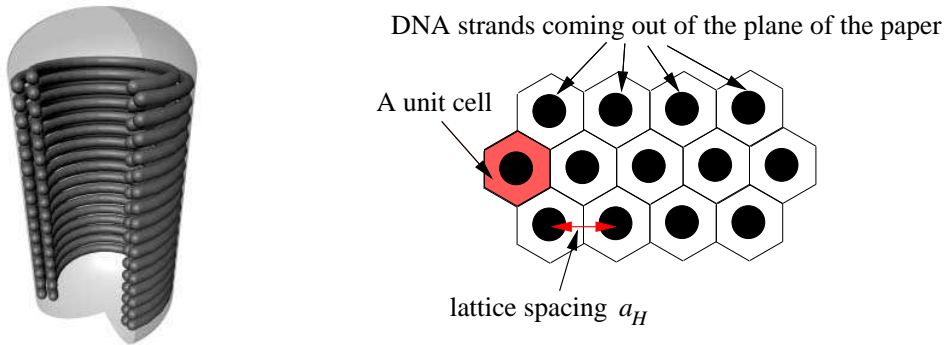
Experiments on the structure of the packaged DNA within phage capsids have a long history: preliminary evidence of hexagonal packaging of the DNA for T2 and T7 phages was first obtained in 1961 [1], and X-ray diffraction was also used to establish that DNA forms concentric layers within the capsid [2–4]. Several theoretical models have been proposed for the topology of the packaged DNA: wound into a spool [49–51], a liquid crystal with hairpins [52, 53] or defects [54], to name but a few, subjecting the topic to much debate. The first unambiguous high-quality images of DNA organization in mature phage capsids were obtained using cryo-electron microscopy in 1997. Averaged over many mature T7 tail-deletion mutant virions, viewed along the portal axis, the packaged DNA showed patterns of circular striations, spaced  $\approx 2.5$  nm apart (Fig. 1) [4]. A computer-modeled projection (side views) of spooled DNA within the capsid, again averaged over many virions, strongly suggested that T7 DNA is wrapped axially in concentric shells of a toroid. In each shell, DNA is coiled with an axial rise of  $\approx 2.5$  nm per turn, and the diameter of each turn is imposed by the previous shell. Following this pioneering study, a succession of cryo-electron microscopy reconstructions have provided support for a toroidal structure:  $\lambda$  [5], both isometric and prolate T4 [6, 7], P22,  $\epsilon$ 15,  $\phi$ 29 [8–13], K1E and K1-5 [14], P-SPP7 [15] and N4 [20] all reveal consecutive layers of toroidal DNA spaced by  $\approx 2.5$  nm. Together, these observations suggest that, when averaged over many virions, the well-defined hexagonal lattice for the packaged DNA may be a generic feature of all phages (Fig. 2). Separately, it should be noted that, where it was resolved in these reconstructions, the leading end (first end to enter the infected cell) is seen to extend into the narrow portal channel. Furthermore, in earlier studies, the leading end of the genome could be cross-linked to the tail of several mature phage virions [16–19]. Portal/tail insertion of the leading DNA end during virion

morphogenesis is also likely a general feature as it ensures that the subsequent DNA ejection step is efficient.



**Figure 1.** Source: Ref. [4]. (b) Cryo-electron images of mature T7 tail-deletion mutant virions, viewed along the portal axis; bar corresponds to 25 nm. (c) Image obtained by averaging over 21 virions, the closed triangle marks the discontinuity between the second and the third DNA-associated rings. (d) Averaging over 77 virions; on the right appears the azimuthally averaged image: S is a dense ring corresponding to the capsid, and 9 other peaks corresponding to 9 DNA rings. Reproduced with permission from Elsevier Inc.

It is of paramount importance to emphasize that the hexagonal toroidal spool structure of the packaged DNA is an *average* property. This point is illustrated beautifully in a recent paper on phage T5 [55]. Further, to be packed within a capsid the DNA helix has to cross itself and therefore cannot lie on a perfect lattice. The toroidal structure has also been suggested to hold only for the part of the DNA close to the inner wall of the capsid, and not for the entire DNA (e.g., Refs. [21, 29]).



**Figure 2.** (Left) Source: Ref. [32]. Idealized DNA packaging in a coaxial toroidal form within the capsid. Reproduced with permission from Elsevier Inc. (Right) Hexagonal lattice formation by the DNA spool, within the capsid. The black filled circles at the center of each hexagonal unit cell (an example is painted pinkish red) denotes the cross-section of the DNA.

### 1.2. Existing approaches for the thermodynamics of packaging and ejection forces

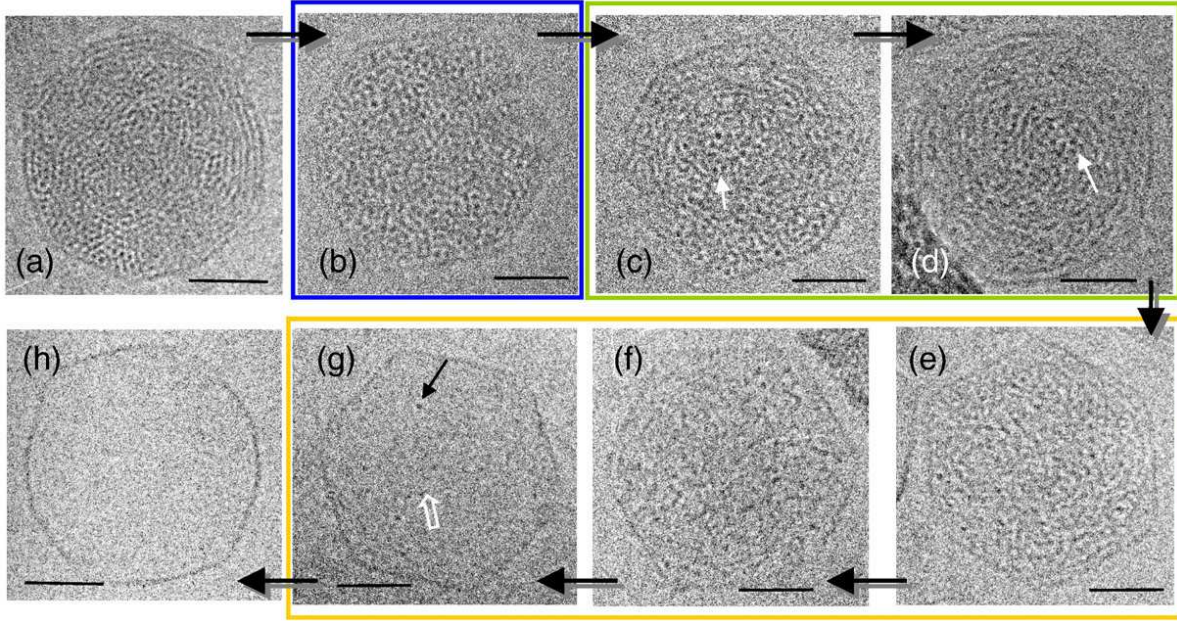
There have been two main — and mutually conflicting — approaches that address the thermodynamic origin of packaging and ejection forces: continuum mechanics and coarse-grained molecular mechanics models. In the continuum mechanics model [31–35],

packaged DNA is seen as a uniformly charged rod, with a certain persistent length  $l_p$ , organized in a toroidal spool on a hexagonal lattice (Fig. 2). The continuum mechanics model is simple enough to allow analytical calculations: packaged DNA is assumed to repulsively self-interact (with electrostatic Coulomb interaction), allowing calculation of the stored electrostatic energy  $U_p^{(el)}$ . Similarly, the bending energy  $U_p^{(b)}$  is obtained from the toroidal spool arrangement of DNA on a hexagonal lattice. Both the electrostatic and bending energies are assumed to be zero for free DNA outside the capsid. Thereafter, with the assumption that the difference between the entropy of free and packaged DNA is negligible, the difference between the Helmholtz free energy of packaged and free DNA is obtained as  $F = U_p^{(el)} + U_p^{(b)}$ . Note that in a given buffered solution and a capsid of volume  $V$ , made of a perfectly rigid material,  $F$  is only a function of the length  $L$  of the DNA within the capsid, i.e.,  $F \equiv F(L)$ . In particular,  $F$  *increases* as a function of  $L$ , and it is  $-\partial F/(A_{DNA}\partial L)$ , the pressure associated with the chemical potential of DNA ejection (the chemical potential of DNA ejection is simply defined as the negative rate of change of the free energy  $F$  with respect to the length of DNA within the capsid) that manifests as the ejection pressure, where  $A_{DNA}$  is the cross sectional area of the DNA. Note that  $\partial F/\partial L$  has the units of force; consequently, in the continuum mechanics model,  $-\partial F/\partial L$  is viewed as the ejection force, and  $\partial F/\partial L$  as the packaging force (note that  $-\partial F/\partial L$  is also the chemical potential of DNA ejection, as defined above).

In the *in vitro*  $\lambda$  experiments [36–39], phage virions are immersed in a solution containing PEG and/or DNA condensing agents, and DNA is ejected when triggered by the LamB receptor protein. The length of the DNA that remains within the capsid at the end of the ejection process depends on the concentration of the PEG and/or DNA condensing agent. (The basic principles underlying these *in vitro* experiments were first presented in a theoretical paper more than four decades ago [25]). The continuum mechanics model has been widely advertised to explain the *in vitro* ejection data, not just for  $\lambda$  but for all phages — both *in vitro* and *in vivo*. A somewhat deeper look, however, reveals that the agreement between theory and the *in vitro* experiments using T5 (one of only three phages studied in osmotic suppression experiments), remains poor [40,41]. The model also neglects how the ejection of proteins from infecting virions (which is a feature of most phages) into the cell is accomplished, and it cannot therefore describe the complete infection process. Further, there is also disagreement between the continuum mechanics theory and the experimental kinetics of T7 DNA translocation into the bacterial cytoplasm *in vivo* [56,57]. All but the leading 0.5 kb of the 70 kb genome of the unrelated phage N4 is also known to be internalized by transcription in the cell [20,42], and marker rescue experiments with phage SP82 suggest that the phage genome enters a cell at a constant rate [43]. None of the *in vivo* experiments that show kinetics of genome internalization are consistent with theories derived from *in vitro* ejection studies.

The continuum mechanics model suffers from fundamentally unrealistic assumptions that diminish its value. In particular, *free energy is an equilibrium concept*; obtaining an ejection pressure by  $-\partial F/(A_{DNA}\partial L)$ , in order to explain the experimental

data on the irreversible ejection process, has the underlying assumption that DNA remains at equilibrium in a toroidal spool configuration on a hexagonal lattice *at all stages of ejection*. Not only is this assumption unsubstantiated by polymer physics theory, it is also inconsistent with actual experimental data on T5 [55]; the latter is illustrated in Fig. 3 below. In order to circumvent some of these problems, the continuum mechanics model invokes two free parameters to fit experimental data.



**Figure 3.** Cryo-EM images of a single T5 virion at different stages of ejection: (a) full capsid to (h) empty capsid, showing the *lack* of toroidal spool configuration of the DNA hexagonal lattice at all stages of ejection, in contrast to the assumption made by the continuum mechanics model. Figure reproduced from Ref. [55], with permission from Elsevier Inc.

In coarse-grained molecular mechanics models [27–30], DNA is modeled as a polymer, with a persistence length  $l_p$ , and polymer dynamics is allowed to take its own course in simulations of phage genome packaging and ejection. The model does not allow analytical calculations — it is effected only by computer simulations, and in work published to date, the DNA is modeled without surrounding water and counterions. However, there is no assumption that DNA remains at equilibrium in a toroidal spool configuration on a hexagonal lattice during packaging or ejection. The main limitation of coarse-grained molecular mechanics model is that because processes are only materialized by computer simulation, they are difficult to correlate with experimental data.

### 1.3. This paper

In summary, our understanding of the thermodynamics of phage DNA packaging and ejection, provided by the existing literature, is far from satisfactory. In this paper

we present a comprehensive treatise of the thermodynamics of packaged DNA and the mechanism of genome ejection, *independent of any specific model*. In Sec. 2 we show that there are four distinct pressures within the capsid: pressure on DNA, hydrostatic pressure, pressure experienced by the capsid, and pressure associated with the chemical potential of DNA ejection. *These are all different thermodynamic quantities*. We also show that for amateur phage the hydrostatic pressure within the capsid is much higher than outside, and that the pressure on the DNA is a lot smaller than commonly envisaged. In Sec. 3 we take up the issue of force transmission along the DNA helix, and show that the pressure on DNA is not important for genome ejection. Instead, in Sec. 4, we suggest that when a phage is triggered *in vitro* to eject by interaction with its receptor, the (thermodynamic) incentive for water to enter the capsid flushes the DNA out. We argue that *in vivo*, the difference between the osmotic pressure within the cell cytoplasm and the outside culture medium initiates, and *importantly maintains*, a flow of water from the culture medium into the capsid, and then from the capsid down the phage tail-tube into the bacterial cell cytoplasm, dragging the DNA out of the capsid and into the cell. We show that this theory is consistent with both studies of *in vitro* ejection and observations of infection *in vivo*.

## 2. Thermodynamics of phages *in vitro*

### 2.1. Entropy of DNA ejection *in vitro*

We consider an experiment with an ensemble of realizations, each containing a phage capsid initially placed in a certain buffer solution: for each realization a DNA of length  $L$  is packaged within a capsid, whose volume we denote by  $V$ . The DNA is allowed to exit the phage *in vitro*, and at the end of the experiment the entire genome ends up in the buffer. The experimental system is kept at a temperature  $T$  at all times. The only interaction of the system with the environment is to exchange (thermal) energy. For the system in its entirety, we define the initial (DNA within the capsid) free-energy  $F_p$  and the entropy  $S_p$ ; the final (DNA in the buffer) free-energy  $F_{\text{unp}}$  and entropy  $S_{\text{unp}}$ . The quantities  $F_{\text{ejection}} = F_{\text{unp}} - F_p$ , and  $S_{\text{ejection}} = S_{\text{unp}} - S_p$  are then the free energy and entropy of ejection, respectively.

The above isothermal experiment, when actually performed in a laboratory, is exothermic [58]. Earlier experiments using differential scanning calorimetry led to the same conclusion [59–61]; i.e., the system releases an amount of heat, of magnitude  $Q_{\text{ejection}}$  to the environment. This tells us that  $S_{\text{ejection}} < 0$ ; however, the amount of heat released to the environment cannot give us the value  $S_{\text{ejection}}$  because the ejection process is spontaneous and irreversible. All we can say, using the second law of thermodynamics, is that  $|S_{\text{ejection}}| \geq Q_{\text{ejection}}/T$ .

The result that the entropy of ejection is negative, from the point of view of DNA confinement in the capsid, is counter-intuitive. One would expect the configurational entropy of the DNA to be strongly reduced due to confinement when compared to the



same DNA in solution; in other words, from the configuration entropy of the DNA in isolation one would expect  $S_{\text{ejection}} > 0$ . This is, in fact, correct; however, the DNA cannot be considered in isolation. The configurational entropy of the DNA is only a very small part of the entropy of the entire system; most of the change in entropy associated with ejection of DNA from its confined state within the capsid into the environment involves the ordering of water molecules around the free DNA [62]. DNA, being a charged molecule, orders the dipole orientation of nearby water molecules. There are many fewer water molecules surrounding the DNA inside the capsid when compared to the same DNA in solution. Consequently, although the DNA molecule does gain entropy following ejection from the capsid, so many more water molecules lose entropy that the entropy of the entire system decreases as DNA is ejected from the capsid.

This issue can be used to evaluate the current approaches for analyzing the physics of phage ejection. DNA configurational entropy is not considered a significant parameter in the continuum mechanics model, while molecular mechanics models [27–30] show that the configurational entropy constitutes a sizable fraction of the free energy of the packaged DNA.

## 2.2. Thermodynamics for the capsid content of a mature phage in a buffer solution

Most phage capsid shells are fully permeable to water and small ions or molecules, but are impermeable to large molecules in the buffer or to the encapsidated DNA. Some phage capsids, like those of T4 and its relatives, are much less permeable, even to small molecules or ions, including cesium chloride and ammonium acetate. Such capsids can easily be broken by osmotic shock, a phenomenon that resulted in the classic electron micrograph showing phage T2 DNA outside a ruptured virion [63].

We assume that the entire system, with DNA packaged within the capsid, can be thermodynamically separated into two subsystems separated by the capsid shell. The subsystem within the capsid consists of DNA (perhaps in addition to protein molecules, which we will not specifically refer to further), and an aqueous environment containing small solutes (including ions) that can permeate the capsid shell, while the subsystem outside usually consists of water and solute molecules that can or cannot permeate the capsid shell. Apart from exchanges of solutes that can permeate the capsid shell, these two subsystems do not interact. This assumption allows us to express  $F_p = F_{p,\text{in}} + F_{p,\text{out}}$ , where  $F_{p,\text{in}}$  and  $F_{p,\text{out}}$  are, respectively, the free energy of the contents of the capsid and that of the environment outside the capsid. Clearly,  $F_{p,\text{in}}$  is a function of the equilibrium capsid volume  $V$ , DNA length  $L$ , number of water molecules  $\{n_{w,\text{in}}\}$  and the number of solute molecules of all species  $\{n_{s,\text{in}}\}$  within the capsid. Similarly,  $F_{p,\text{out}}$  is a function of number of water molecules  $\{n_{w,\text{out}}\}$  and the number of solute molecules of all species  $\{n_{s,\text{out}}\}$  in the solution. Both subsystems are “open” in the thermodynamic sense, since they exchange water and small solute molecules. We assume (realistically) that the volume of the buffer in which the phage is immersed is  $\gg V$ , so for analyzing the thermodynamics of the phage and its contents, the external solution can



be considered to be an infinite reservoir for water and small permeable solute molecules. The number of solute molecules within the phage capsid  $\{n_{s,\text{in}}\}$  is then fixed by the chemical potential of the small solute molecules in the buffer, collectively denoted by  $\{\mu_s\}$  (for the case of counterions, via the Donnan equilibrium). This simply means that  $F_{\text{p,in}} \equiv F_{\text{p,in}}(V, L)|_{\{\mu_s\}}$ . The free energy  $F_{\text{p,in}}(V, L)|_{\{\mu_s\}}$  can be further dissociated into the interaction free energy  $F^{(\text{int})}(V, L)|_{\{\mu_s\}}$  of the ions, DNA and water molecules within the capsid, and the DNA configurational free energy (this includes bending, or curvature, energy and the configurational entropy of the DNA)  $F_c(V, L)|_{\{\mu_s\}}$  of the DNA is then given by

$$F_{\text{p,in}}(V, L)|_{\{\mu_s\}} = F_{\text{p}}^{(\text{int})}(V, L)|_{\{\mu_s\}} + F_c(V, L)|_{\{\mu_s\}}. \quad (1)$$

We also assume that water is incompressible and that small solutes do not occupy any physical volume. With these assumptions, in equilibrium, the number of water molecules within the capsid is then determined by  $V$  minus the physical volume of the DNA, and we can now explicitly focus on a number of thermodynamic issues.

*2.2.1. Pressure associated with the chemical potential for DNA ejection* If we assume that the capsid is made of a perfectly rigid material, then from the above definitions  $F_{\text{p,in}}(V, L)|_{\{\mu_s\}} \equiv F_{\text{p,in}}(L)|_{\{\mu_s\}}$ , we can define the pressure associated with the chemical potential for DNA ejection. Consider two situations using the same buffer conditions (i.e., at fixed chemical potentials of permeable ions), one where DNA of length  $L$  and the other a length of  $L - dL$ , is packaged within the capsid. We assume that Donnan equilibrium conditions are satisfied for both lengths of DNA within the phage capsid. One can then define the pressure associated with the chemical potential of DNA ejection by comparing the free energy of the capsid content under these two situations, viz.  $F_{\text{p,in}}(L)|_{\{\mu_s\}}$  and  $F_{\text{p,in}}(L - dL)|_{\{\mu_s\}}$ , as:

$$P_{\text{ejection}} = -\frac{1}{A_{\text{DNA}}} \frac{\partial F_{\text{p,in}}(L)|_{\{\mu_s\}}}{\partial L}, \quad (2)$$

where  $A_{\text{DNA}}$  is the cross-sectional area of the DNA. The pressure  $P_{\text{ejection}}$  is the pressure that the plug at the end of the tail tube feels from within the phage.

*2.3. Pressure on the capsid from within, hydrostatic pressure imbalance across the capsid and pressure on the DNA*

In Sec. 2.2, we considered the thermodynamics of the capsid content as a subsystem, under a fixed chemical potential of permeable small solute molecules in the buffer, while assuming that the number of water molecules within the capsid is simply determined by the capsid volume. This is however an incomplete description of the thermodynamics of the entire system (namely the phage immersed in the buffer solution), as we did not consider the thermodynamic penalties associated with exchange of water molecules across the capsid shell. If the capsid is made of a perfectly rigid material without the possibility of expansion or contraction, such a description would be correct, but in

reality, the capsid is comprised of protein molecules, and therefore has a finite, albeit high, rigidity [65]. Keeping the assumption that water is incompressible, rather than a fixed capsid volume determining the number of water molecules internalized, the capsid volume should actually be determined by the thermodynamics of water molecule exchange across the capsid shell, which is equivalent to a semi-permeable membrane.

We now argue that for a given length  $L$  of the DNA within the capsid, the equilibrium volume  $V$  is determined by the buffer composition. We presuppose that an expansion/contraction of the capsid allows for an exchange of a small number of water molecules, relative to the total number of water molecules present in the external buffer. We can then safely assume that the chemical potential of permeable small solutes in the buffer remains unaltered by the exchange of water molecules across the capsid shell.

First, the pressure on the capsid from within,  $P_{\text{capsid}}$ , can be calculated from the  $V$ -dependence of the free energy  $F_{\text{p,in}}(V, L)|_{\{\mu_s\}}$  as follows. Imagine a virtual, uniform expansion of the capsid leading to a volume increase from  $V$  to  $V + dV$  (the extra volume will be filled by water from the buffer and permeable solute molecules as dictated by  $\{\mu_s\}$  — for the case of ions, via the Donnan equilibrium). For a given length of DNA, under fixed  $\{\mu_s\}$ , this leads to a new value of the free energy of the inside material of the capsid,  $F_{\text{p,in}}(V + dV, L)|_{\{\mu_s\}}$ , but also, since the buffer solution loses a volume  $dV$  of water, the free energy of the buffer solution increases by an amount  $\pi_{\text{out}}dV$ , where  $\pi_{\text{out}}$  is the osmotic pressure of the buffer solution. The pressure on the capsid from within, is therefore given by

$$P_{\text{capsid}} = -\frac{\partial F_{\text{p,in}}(V, L)|_{\{\mu_s\}}}{\partial V} - \pi_{\text{out}}. \quad (3)$$

Secondly, water's thermodynamic incentive to enter the capsid is given by the osmotic gradient across the capsid shell, namely  $[\pi_{\text{in}} - \pi_{\text{out}}]$ , where  $\pi_{\text{in}}$  is the osmotic pressure within the capsid shell, and is defined by [see Eq. (1)]

$$\pi_{\text{in}} = -\frac{\partial F_{\text{p}}^{(\text{int})}(V, L)|_{\{\mu_s\}}}{\partial V}. \quad (4)$$

Simultaneously, when entering the capsid, the water molecules exit a zone of hydrostatic pressure  $P_{\text{hydro,out}}$ , the hydrostatic pressure in the solution outside the capsid, and enter the capsid which may have, in principle, a different pressure,  $P_{\text{hydro,in}}$ . The equilibrium capsid volume is then determined by the condition that  $[\pi_{\text{in}} - \pi_{\text{out}}]$  is counter-balanced by the pressure-volume work done by water molecules in entering the capsid

$$P_{\text{hydro,in}} - P_{\text{hydro,out}} = \pi_{\text{in}} - \pi_{\text{out}}. \quad (5)$$

It is interesting to note that Eq. (5) is well-known to hold for bacterial and plant cells: the osmotic pressure gradient is counter-balanced by a hydrostatic pressure differential (turgor) [68]. Turgor allows cells to enlarge and thus facilitates growth.

Further, an appreciation for Eq. (5) is afforded by a straightforward gedanken experiment that connects to elementary physical chemistry. Consider a vertical U-tube with a membrane at the lowest point separating the two arms. The membrane is

permeable to water, but impermeable to, say, sugar molecules. We fill up the two arms of the U-tube to equal height: the left arm with pure water, and the right arm with sugar solution. As time progresses, water from the left arm will permeate into the right arm, reducing the height of the water column on the left, while increasing the height of the sugar solution column on the right. At equilibrium, the height of water in the left column will be lower than the sugar solution, meaning that there is a hydrostatic pressure difference  $P$  across the membrane. Moreover, at equilibrium the concentration of sugar in the right column is non-zero, while it remains zero in the left column by construction. There is therefore an osmotic gradient across the membrane, which is precisely counter-balanced by the hydrostatic pressure  $P$ . Equation (5) describes the same equilibrium, where the difference between the osmotic pressures inside and outside the capsid is counter-balanced by the hydrostatic pressure difference across its protein shell.

Equations (3) and (5) allow us to appreciate not only how the pressure on the capsid of finite rigidity is mechanically materialized, but also how the pressure  $P_{\text{DNA}}$  that the capsid mechanically transmits to the DNA (and by action-reaction, the pressure the DNA transmits to the capsid). First, the force balance on a small capsid surface tells us that the capsid pressure  $P_{\text{capsid}}$  equals the hydrostatic pressure gradient across the capsid plus the pressure that is mechanically transmitted to the DNA inside; i.e.,

$$P_{\text{capsid}} = P_{\text{hydro,in}} - P_{\text{hydro,out}} + P_{\text{DNA}}. \quad (6)$$

Incorporating Eqs. (1) and (4-6), this reads

$$P_{\text{DNA}} = -\frac{\partial F_{\text{p,in}}(V, L)|_{\{\mu_s\}, L}}{\partial V} - \pi_{\text{in}} = -\frac{\partial F_c(V, L)|_{\{\mu_s\}}}{\partial V}. \quad (7)$$

Note in Eq. (7) that the osmotic pressure within the capsid  $\pi_{\text{in}}$  is derived from the interaction energy of the ions, DNA and water molecules within the capsid, while the same interaction energy appears in  $F_{\text{p,in}}(V, L)|_{\{\mu_s\}}$ , but with opposite sign [Eq. (4)]. Consequently, the net contribution of this interaction energy to the r.h.s. of Eq. (7) is zero. This conclusion implies that the pressure on the DNA mechanically transmitted by the capsid comes entirely from the stiffness of the DNA. We verify this conclusion in Sec. 3 for a single turn of the DNA. Therein we also discuss why Eq. (7) is not in conflict with the experiment by Smith *et al.* [26].

It is straightforward to show that Eqs. (3-7) are consistent with the Gibbs free energy minimization of the entire system (the mature phage plus the buffer solution). Moreover, we note that due to the cancellation of the interaction energy term in Eq. (7) as explained above, the compressive forces on the DNA mechanically transmitted by the capsid are small compared to  $P_{\text{capsid}}$ : the bulk of  $P_{\text{capsid}}$  is mechanically transmitted to the water inside. This is in fact confirmed from the structural and interaction properties of both the packaged DNA and the structure of the protein molecules comprising the capsid shell. Raman spectral studies of mature P22 and T7 phages have not found any evidence of any structural alteration of protein or DNA that would be expected if they were subjected to high pressure [66, 67]. Only the configuration of the phosphodiester

groups in packaged DNA are perturbed from that found in free DNA solutions, and the capsid protein structures of mature virions are indistinguishable from empty, DNA-free, particles.

Finally, we note from Eqs. (2-7) that the osmotic pressure  $\pi_{\text{in}}$  within the capsid, the pressure  $P_{\text{ejection}}$  associated with the chemical potential of DNA ejection, and the pressure  $P_{\text{DNA}}$  imparted by the capsid on the DNA are entirely different thermodynamic quantities. Importantly, it must be appreciated that these pressures are all equilibrium quantities, and they cannot be used to describe a non-equilibrium situation.

### 3. Can $P_{\text{DNA}}$ and $P_{\text{ejection}}$ play any role in DNA ejection dynamics?

In this section, we investigate whether  $P_{\text{DNA}}$  and  $P_{\text{ejection}}$  play a role in DNA ejection. We first discuss the case of  $P_{\text{DNA}}$  within the context of the continuum mechanics model; in order to do so we start by analyzing the stability of the DNA within a phage capsid.

#### 3.1. Stability of the DNA within a phage capsid

As described in Secs. 1.2, continuum mechanics model starts with the assumption that the DNA, within a capsid made of a perfectly rigid material, takes a toroidal spool configuration on a hexagonal lattice at all stages of ejection. Given that the outer radius of the spool is fixed by the capsid dimensions, all structural aspects of the spool are then determined by the spacing of the hexagonal lattice. Phages with internal cores constitute a special case: the radius of the innermost ring of the spool is simply given by the radius of the core. For a given ionic condition  $\{n_{\text{s,in}}\}$  inside the capsid — which in turn is fixed by the chemical potential  $\{\mu_{\text{s}}\}$  of the small solutes in the external buffer (for the case of counterions, via the Donnan equilibrium) — and DNA of length  $L$  within a capsid of volume  $V$ , the free energy of the material inside the capsid is a function of  $a_H$  alone, where  $a_H$  is the hexagonal lattice spacing; i.e.,  $F_{\text{p,in}}(V, L)|_{\{\mu_{\text{s}}\}} \equiv F(a_H)|_{\{\mu_{\text{s}}\}}$ . Note that the configurational entropy of the DNA is not a meaningful quantity in the continuum mechanics model, hence in this section  $F_c$  only contains curvature (bending) energy of the DNA; i.e.,  $F_c(V, L)|_{\{\mu_{\text{s}}\}} \equiv U_c(a_H)$ .

Within the continuum mechanics model, one can argue that  $a_H$  is fixed, via a trade-off between  $F^{(\text{int})}(a_H)|_{\{\mu_{\text{s}}\}}$  and  $U_c(a_H)$  [see Eq. (1)]. The interaction free energy can be lowered by increasing  $a_H$ . However, since the “volume constraint” (meaning that the total available volume within the capsid is  $V$ ) must be obeyed, increasing  $a_H$  will reduce the minimum radius of the DNA spool, because within the inner part of the spool the DNA will be much more tightly bent, which will increase its bending energy. In this manner, a reduction in interaction free energy increases curvature energy and *vice versa*. In other words the trade-off between these two quantities dictates that:

$$\frac{\partial F^{(\text{int})}(a_H)|_{\{\mu_{\text{s}}\}}}{\partial a_H} + \frac{\partial U_c(a_H)}{\partial a_H} = 0, \quad (8)$$

which minimizes the free energy  $F(a_H)|_{\{\mu_{\text{s}}\}}$ . We denote the value of  $a_H$ , obtained from

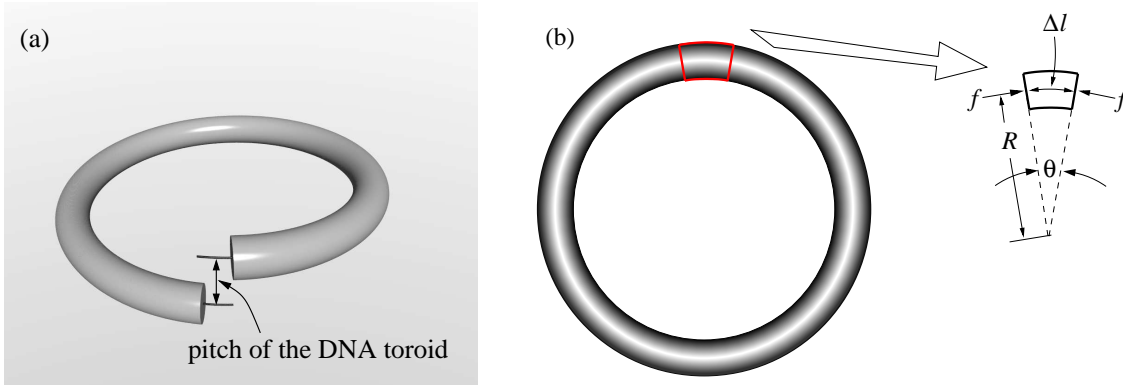
Eq. (8) by  $a_H^{(0)}$ , and the corresponding minimum of the free energy of the inside content of the capsid by  $F_{p,in}^{(0)}|_{\{\mu_s\}} \equiv F(a_H^{(0)})|_{\{\mu_s\}}$ . If it is further assumed that most of the interaction free energy variation due to variations in  $a_H^{(0)}$ , caused by a virtual uniform expansion of the capsid [as in Eq. (4)], involves the DNA helix, then the osmotic pressure within the capsid can also be defined as

$$\pi_{in} = -\frac{2}{3\sqrt{3}a_H^{(0)}L} \frac{\partial F^{(int)}(a_H^{(0)})|_{\{\mu_s\}}}{\partial a_H^{(0)}}, \quad (9)$$

where the volume that the DNA toroid occupies within the hexagonal lattice is given by  $3\sqrt{3} \left[ a_H^{(0)} \right]^2 L/4$ . This analysis for the specific case of the phage T7 — a phage with an internal proteinaceous core — can be found in Ref. [64].

### 3.2. Can $P_{DNA}$ transmit down the tail tube?

The only way  $P_{DNA}$  can play a role in DNA ejection is if it can transmit from within the capsid, where the bulk of the DNA is located, down the tail tube. To evaluate this idea, we begin with the appreciation that  $P_{DNA}$  favors a uniform expansion of the capsid. However, since DNA is not a continuum material,  $P_{DNA}$  does not trivially transmit along the helical axis into the tail tube. Nevertheless, when the DNA organization within the capsid is averaged over many phage particles, the picture that emerges is that pressure on the toroidal spool of DNA does translate into a *compressive* force  $f$  acting along the contour of the DNA. Following Sec. 3.1, we start by analyzing this force within the continuum mechanics model, and we then ask whether this force can transmit down the tail tube.



**Figure 4.** (a) One full turn of the DNA toroid around the axis of the idealized toroidal spool in Fig. 2. (b) With the pitch ignored: the corresponding DNA ring of radius  $R$ , and the compressive force  $f$  along the contour of the DNA, shown on a differential length element  $\Delta l$  of the ring.

We consider one full turn of DNA around the axis of the idealized toroidal spool [Fig. 4(a)]. For simplicity, we ignore the pitch of this turn, i.e., it is idealized as a DNA ring of radius  $R$ , and we focus on a differential length segment  $\Delta l$ . The DNA ring and

the compressive force along the DNA contour are shown in Fig. 4(b). The fact that the entire DNA spool is under a pressure  $P_{\text{DNA}}$  within the phage capsid implies that the isolated ring itself is under a pressure  $P$ , but whose relation to  $P_{\text{DNA}}$  we have not specified.

Within the scheme of continuum mechanics, the force balance equation for this differential length segment along the transverse direction (along a line joining the differential length element and the center of the ring) is given by

$$f \sin \theta \approx f \theta = P \Delta l t, \quad (10)$$

where  $t$  is the cross-sectional diameter of the DNA. Further, the geometrical consideration from Fig. 4(b) tells us that

$$\sin \theta \approx \theta = \Delta l / R, \quad (11)$$

i.e.,

$$f = P R t. \quad (12)$$

We now note that, just like in Eq. (8), the DNA ring stays in its configuration by the balance of two competing thermodynamic forces, one derived from its curvature energy  $u_c$ , the other from the energy of self-interaction with nearby DNA strands. The thermodynamic force related to curvature energy favors an increase of the radius of the ring, while the thermodynamic force related to the self-interaction free energy favors a decrease. The magnitude of this force is given by

$$(2\pi R) P t = -\frac{\partial u_c}{\partial R} = \frac{2\pi(k_B T)l_p}{R^2}, \quad (13)$$

implying that

$$f = P R t = \frac{(k_B T)l_p}{R^2}. \quad (14)$$

At room temperature ( $T = 300$  K)  $k_B T = 4.14 \times 10^{-21}$  Joules. If we take  $l_p = 50$  nm, the widely used value, then

$$f = \frac{2.07 \times 10^{-28}}{R^2} \text{ Nm}^2. \quad (15)$$

Thus, depending on the radius of the DNA ring, the magnitude of the compressive force  $f$  along the contour of the DNA can be a few pN. Equation (15) tells us that the pressure on this DNA ring is only in terms of the stiffness of the DNA, as we argued in Sec. 2.3.

How much of this force can transmit down the tail tube? We should remember that force is a vector quantity. Although the above exercise shows that there is a compressive force along the contour of the DNA ring that make up the toroidal spool, it is necessary to follow the actual turns of the DNA leading into the tail tube in order to identify how much of the force  $f$  is actually being transmitted. However, the definition of persistence length  $l_p$  in polymer physics means that while a DNA molecule of length  $\lesssim l_p$  is semi-flexible, a DNA molecule with length  $\gg l_p$  is simply a flexible polymer. It is therefore impossible to transmit compressive forces along the contour of a DNA that is  $\gg 50$  nm. This consideration alone ensures that any compressive force along the contour of, e.g.,

an  $\sim 16 \mu\text{m}$  long  $\lambda$  genome will not transmit into the tail tube. Therefore, compressive forces on the packaged phage genomic DNA cannot be important for DNA ejection.

Our estimate of  $f$  having a magnitude of a few pN is, at first blush, in apparent contradiction with the experiment by Smith *et al.* [26]. A deeper look however immediately reveals that Eq. (15) and the forces exerted by the packaging motor are actually compatible. Thermodynamically, we have identified four different pressures within the capsid: pressure on the DNA  $P_{\text{DNA}}$ , hydrostatic pressure  $P_{\text{hydro,in}}$ , the pressure experienced by the capsid  $P_{\text{capsid}}$ , and the pressure  $P_{\text{ejection}}$  associated with the chemical potential of DNA ejection. These are all different quantities. Smith *et al.* [26] measure  $P_{\text{ejection}}$ ; they assumed that the work done against  $P_{\text{ejection}}$  by the packaging motor is used to pressurize the DNA and is conserved in the capsid. However, most of the work done against  $P_{\text{ejection}}$  by the packaging motor is expended in increasing the osmotic pressure within the capsid. This increase is due to the expulsion of water molecules out of the capsid — a reverse osmosis process — in order to allow the phage genome being packaged to condense, while only a small part of the work is used to increase  $P_{\text{DNA}}$ . The energy expended during water expulsion is not conserved by the packaged genome, which explains our lower estimate of  $P_{\text{DNA}}$  inside the capsid. In this context it is important to remember that osmotic pressure is only a measure for the chemical potential of water: the fact that the osmotic pressure inside the capsid is high does not imply that the DNA is under enhanced pressure.

### 3.3. Is $P_{\text{ejection}}$ important for DNA ejection dynamics?

Equation (2) shows that for a mature phage in a given buffer, the tail plug feels a thermodynamic pressure  $P_{\text{ejection}}$  from inside the capsid. Given that  $P_{\text{ejection}}$  is a thermodynamically derived quantity (i.e., derivative of  $F_{\text{p,in}}$  with respect to  $L$ ), it is clear that if genome ejection were a quasi-equilibrium process, it would indeed govern the ejection dynamics. However, the underlying assumption of quasi-equilibrium is that at all stages of ejection the DNA remaining within the phage head stays in a toroidal loop, with an ever-increasing spacing between the toroidal strands as ejection proceeds. This is the major thesis of the continuum mechanics model, which uses  $P_{\text{ejection}}$  to explain the *in vitro* experimental data using phage  $\lambda$ .

Whether this thesis and its underlying assumption are correct or not lies in the question of time scales. If the time-scales associated with the equilibration of DNA (in the form of a toroidal spool on a hexagonal lattice) and the small solute molecules within the phage capsid (maintained at a fixed chemical potential by the external buffer), are much smaller than the ejection time, equilibrium pressures and forces can be used to describe genome ejection. However, recent experimental evidence with T5 clearly demonstrates that as the DNA leaves the capsid, it goes through a series of phase transitions [55]. The cryo-electron microscopic images of T5 virions during DNA ejection strongly suggest that the process is non-equilibrium, which invalidates the use of  $P_{\text{ejection}}$  to describe its dynamics. Comparable experiments have not yet been conducted with



other phages, but there is no justifiable reason to assume that the genome of other phages, in particular those lacking a defined internal core structure, whose DNA is packaged to the same density, would behave otherwise. We are not aware of experimental studies that probe the equilibration time-scales of confined DNA, such as within a phage capsid. However, the high velocity of phage DNA ejection measured *in vitro*: up to 75 kb/sec (T5) with long pauses between distinct steps [44], and up to 60 kb/sec ( $\lambda$ ) in 10 mM  $\text{Na}^+$  buffer or  $\sim 20$  kb/sec in 10 mM  $\text{Mg}^{2+}$  [45], makes it highly unlikely that these are quasi-equilibrium processes. These experimental data suggest that  $P_{\text{ejection}}$  cannot be used to describe DNA ejection dynamics even *in vitro*.

#### 4. An alternative mechanism of DNA ejection from phage virions

Sections 2 and 3 illustrate the fundamental problems with the idea that the thermodynamically derived ejection pressure  $P_{\text{ejection}}$  (or the corresponding ejection force  $A_{\text{DNA}}P_{\text{ejection}}$ ) causes DNA ejection from phages. There is therefore a clear need for a mechanism that can explain the physics of DNA ejection, one that is consistent with both *in vitro* and *in vivo* experimental data. We suggest such a mechanism below.

##### 4.1. DNA ejection *in vitro*

Of the two main approaches for DNA ejection: continuum mechanics and coarse-grained molecular mechanics models, only the former addresses, using  $P_{\text{ejection}}$ , the DNA ejection mechanism in a way that can be compared to *in vitro* experimental data. In these creative experiments, phages are immersed in a solution containing PEG and/or DNA condensing agents, and DNA is ejected from the virion when triggered by the receptor protein. The length of DNA that remains within the capsid at the end of the ejection process depends on the concentration of the PEG and/or DNA condensing agent.

With the caveats we have discussed concerning the validity of using  $P_{\text{ejection}}$  to describe phage DNA ejection, and remembering that  $P_{\text{ejection}}$  also involves fitted parameters, the continuum mechanics theory does provide good agreement with *in vitro* genome ejection data for  $\lambda$  and SPP1 (e.g., Refs. [36–38, 47, 48]). However, the data obtained with just two phage systems, along with the physics of the continuum mechanics model, have often been extrapolated to all phages and in addition to explain all phage DNA ejection *in vivo*. These generalizations ignore data from the third phage system that has been studied *in vitro*, which provides rather different conclusions. At the presumed pressures internal to the largely full T5 capsid, *in vitro* experimental data can be fitted to the continuum mechanics model, but at low to moderate pressures, when approximately half or less of the genome remain in the capsid, multiple populations are found to co-exist [40]: some phages have completely ejected their genomes, whereas others have ejected a varying amount of DNA that is not dependent on the external osmotic pressure. Furthermore, at  $\leq 2$  atm external osmotic pressure, most T5 virions completely eject their DNA. In contrast, the same external pressure prevents ejection

of  $\sim 40\%$  of the  $\lambda$  genome [37]. Theories dependent on  $P_{\text{ejection}}$  are clearly unable to explain how *all* phages eject their DNA, even *in vitro*.

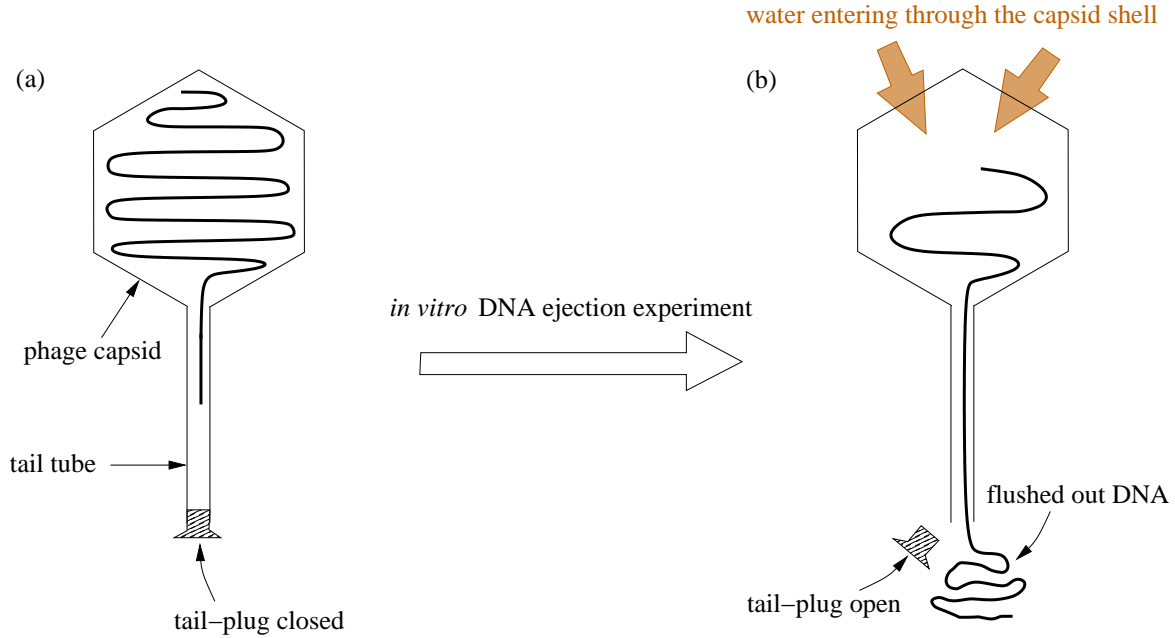
*4.1.1. Hydrodynamic model of phage DNA ejection* As discussed in Sec. 3.3, in an *in vitro* experiment when a mature phage is equilibrated in a certain buffer solution, the difference between  $\pi_{\text{in}}$  and the osmotic pressure  $\pi_{\text{out}}$  of the buffer solution determines the thermodynamic incentive for water molecules to enter the capsid. Equation (5) shows that if the phage tail is plugged, this incentive is counter-balanced by a hydrostatic pressure gradient across the capsid shell (and the capsid, being of high but not infinite rigidity, will be slightly expanded from its relaxed state under the enhanced hydrostatic pressure from inside). We propose that it is this thermodynamic incentive of water molecules to enter the capsid that flushes the DNA out when the tail plug is opened by the action of the appropriate receptor. We refer to this idea as the hydrodynamic model of DNA ejection.

When the tail plug is opened by the appropriate receptor, e.g., LamB protein in the case of  $\lambda$ , FhuA for T5, or YueB780 for SPP1, it is likely that the excess hydrostatic pressure within the capsid is partially relieved by a transient water flow down the tail tube and, as a result, the capsid will reduce in size. Although water flow down the tail-tube will exert a hydrodynamic force that is likely to drag along the DNA, one end of which is already inserted into the tail-tube, this process will not last long enough to drag the entire genome out of the virion. While a very small contraction of the capsid is unlikely to be detected even by state-of-the-art experiments, we can provide a “guesstimate” for how much DNA could be dragged out by this transient water release. With a typical capsid diameter  $\sim 60$  nm (assumed spherical), let the capsid diameter contract by 0.1 nm; the water released is  $4\pi(30)^2(0.05)$  nm<sup>3</sup>. With a typical tail tube inner diameter  $\sim 4$  nm, its cross-sectional area is  $\pi(2^2)$  nm<sup>2</sup> (ignoring the cross-section of the DNA within the tail tube) and the length of the water column passing into the tail-tube  $= 4\pi(30^2)(0.05)/[\pi(2^2)]$  nm = 45 nm. Assuming the DNA flows freely with this water column down the tail, in this example the DNA, which occupies 25% of the cross-sectional area of the tube, can be moved 60 nm, corresponding to  $\sim 180$  base pair of a phage genome.

Once the water pressure difference across the capsid shell is relieved by this transient water flow, so long as the difference between the osmotic pressure of the remaining DNA within the capsid and  $\pi_{\text{out}}$  stays positive, water will have a thermodynamic incentive to move into the capsid using whichever path it can find. However, since the capsid has a finite volume, any water movement into the capsid can only occur at the expense of an equal volume of water, now containing DNA (as a solute) being ejected through the tail tube. In other words, following the initial transient water release down the tail-tube, continued water movement from the buffer into the capsid up an osmotic gradient will cause the DNA to be ejected. This process will stop when the osmotic pressure of the leftover amount of DNA in the capsid equals  $\pi_{\text{out}}$ . The physics behind this *in vitro* DNA ejection experiment (omitting the transient water flow down the tail tube due to the

excess hydrostatic pressure) is schematically shown in Fig. 5.

The hydrodynamic mechanism for DNA ejection commensurate with *in vitro* experimental data. All that is required to drive DNA ejection is an incentive for the water molecules to enter the capsid, which is decided dynamically in a non-equilibrium manner. There is no *a priori* assumption that phage genome ejection is a quasi-equilibrium process, and thus the model is not dependent on thermodynamic analyses.



**Figure 5.** Schematic of an *in vitro* DNA ejection experiment. (a) Mature phage equilibrated in a certain buffer solution. The difference between  $\pi_{\text{in}}$ , the pressure on the DNA, and the osmotic pressure  $\pi_{\text{out}}$  of the buffer solution is counter-balanced by a hydrostatic pressure difference across the capsid shell [Eq. (5)]. (b) The tail plug is opened by the action of the receptor, following any transient water release down the tail-tube (not shown), water enters the phage capsid and flushes the DNA into the buffer.

The difference between the osmotic pressure of the residual DNA in the capsid and  $\pi_{\text{out}}$  is controlled by two aspects: (i) the presence of any small DNA condensing agents that reduces the osmotic pressure of the capsid content without altering  $\pi_{\text{out}}$ , and (ii) the presence in the external buffer of large solute molecules like PEG that increase  $\pi_{\text{out}}$ , but do not affect the inside osmotic pressure as they cannot penetrate into the capsid. Both achieve the same thermodynamic incentive differential for water between inside and outside the capsid, and are qualitatively in agreement with *in vitro* DNA ejection data [36–41, 44–48]. When the osmotic pressure of any DNA remaining in the capsid  $= \pi_{\text{out}}$  (such as at the end of the ejection process), there will be no further flow of water into the capsid, and thus no more DNA ejection.

The hydrodynamic model requires that the time-scale of water flow into the capsid

be sufficiently fast to match the time-scales for DNA ejection *in vitro*. To this end, we refer to an experiment where a mature wild-type  $\lambda$  capsid was locally deformed (compressed) by up to 25% of its original volume with an AFM tip [65]. Compression of the capsid without it rupturing has to drive out internal water and small solutes. When the AFM tip was removed, the virion capsid returned to its normal size and shape within  $\sim 4$  ms. This suggests that the large surface area to volume ratio of the capsid allows copious amounts of water to diffuse — a slow process relative to hydrodynamic processes — across the capsid shell in a very short time. Extrapolating these data to yield a complete exchange of the contents of the capsid with the external buffer gives 20 ms; assuming that DNA moves freely, this value results in a rate of phage genome ejection *in vitro* at least 50 times the maximum measured to date.

By itself, the hydrodynamic model cannot explain either the stepwise ejection of DNA from T5 particles or the coexistence of virions containing different amounts of DNA at low external pressures. A partial *ad hoc* solution for T5 (one that is also necessary for the  $P_{\text{ejection}}$  model of DNA ejection) is that the tail tube becomes temporarily blocked by protein conformational changes, thereby impeding water flow — and thus stopping DNA ejection. Note that water flow from the external buffer into the capsid interior will be simultaneously impeded. Release of the blockage by a further conformational change in the tail proteins then allows resumption of water flow and thus of DNA ejection. Interestingly, the apparent activation energy for releasing the block in the step-wise ejection process of T5 DNA ejection has been experimentally shown to be independent of the amount of DNA remaining in the capsid [37, 70].

In summary, the hydrodynamic model is fully compatible with the experimental data for  $\lambda$  and for SPP1 DNA ejection *in vitro*. Like thermodynamics-based models (e.g., continuum mechanics or molecular dynamics), the hydrodynamic model requires an *ad hoc* assumption to explain the step-wise process of T5 DNA ejection. However, the hydrodynamic model is not dependent on the critical assumption — used in  $P_{\text{ejection}}$ -based models — that the encapsidated DNA remains at equilibrium at all times during the ejection process. Furthermore, as we argue below, only the hydrodynamics model explains how complete genome ejection can be achieved in the face of an opposing force. This is the situation in natural infections of bacterial cells.

## 4.2. DNA ejection *in vivo*

Before discussing specific mechanisms of phage DNA ejection *in vivo*, it is instructive to consider some salient points about phage infections.

**4.2.1. Phage infection of bacteria** Largely because of influential textbooks and, until recently, of only little experimental information, it is not widely appreciated that *all* phages eject proteins into the cell [69, 71]. At a minimum, the protein(s) comprising the tail plug must be removed, and for long-tailed phages (like T4, T5, SPP1 and  $\lambda$ ) the tape measure protein, which determines the precise length of the tail, must be ejected

in order to allow the phage genome to pass through the tail tube. Short tailed phages may eject internal proteins into the cell to extend — at least functionally — their tail so that it can span the infected cell envelope [57].

Some phages eject many different protein molecules from their capsid, some necessarily before, others perhaps after, genome ejection. To give just two examples, T7 virions ejects  $\sim 75$  molecules representing five different protein species into the cell prior to DNA penetration of the cell cytoplasm [72], and T4 virions eject  $\sim 1000$  IP (internal protein) molecules into the cell [73]. Clearly, any model purporting to explain even dsDNA phage genome ejection *in vivo* must also accommodate ejection of virion proteins.

Phage virions can also eject more than one DNA molecule [74,75]. However, as the diameter of the channel through the portal complex and tail tube (inner diameter  $\sim 40$  Å) cannot accommodate more than a single DNA helix, the leading end of the second (and perhaps subsequent molecules) must find the exit channel without a built-in guiding vectorial force. Genome ejection by single-stranded DNA (ssDNA) or RNA viruses, whose nucleic acid is packaged at much lower densities than dsDNA phages, should also be explained by models describing phage DNA ejection. Packaged genomes of these phages are not thought to be under pressure, and their mode of genome ejection has therefore not been considered by continuum mechanics or molecular mechanics models.

The *E. coli* cytoplasm has a positive osmotic pressure of several atm above the environment (under various growth conditions the pressure has been estimated to vary between 2 and 15 atm, with 3.5-5 atm being commonly accepted values [76,77]). This osmotic pressure gradient is counter-balanced by a hydrostatic pressure differential (turgor) [68] that enables the cell to enlarge during growth [78]. Gram-positive cells, such as *Bacillus subtilis*, the host for phage SPP1, have much higher turgor:  $\sim 19$  atm [79]. If turgor is assumed to provide an opposing force to phage genome ejection into the cytoplasm, then a  $P_{\text{ejection}}$ -based mechanism cannot possibly explain complete genome ejection into cells. This problem was recognized in the first osmotic suppression of genome ejection experiments *in vitro* [38], but has often been ignored in subsequent general statements about how phages infect cells *in vivo*. Interestingly, the osmotic pressure of the cytoplasm declines as bacteria enter stationary phase, which should allow better genome penetration by a  $P_{\text{ejection}}$ -based ejection mechanism. However, stationary phase bacteria are not readily infected by most phages [94], which infect exponentially growing cells, with their higher turgor pressure, much more efficiently.

A bacterial cytoplasmic membrane is composed of a phospholipid bilayer that is impermeable to most molecules other than water and glycerol. An electrical potential is maintained across this membrane. A strong osmotic gradient also exists between the external medium and the cell cytoplasm. The higher internal pressure (turgor) is necessary for bacteria to enlarge and undergo cell division. In addition, at all times, a  $K^+$  concentration gradient  $[K^+]$ , with  $[K^+]$  high inside, is maintained in cells (a reverse gradient of  $Na^+$  concentration also usually exists). These conditions are perturbed when a bacterium is infected by a phage as a direct connection between the cytoplasm and

the external medium is opened. This connection passes through the phage capsid and tail, and if it is open, and, as we have argued, water will flow from the external medium into the cell to neutralize the overall osmotic gradient. Simultaneously,  $K^+$  will flow from the cytoplasm into the external medium. Furthermore, the membrane potential can no longer be maintained.

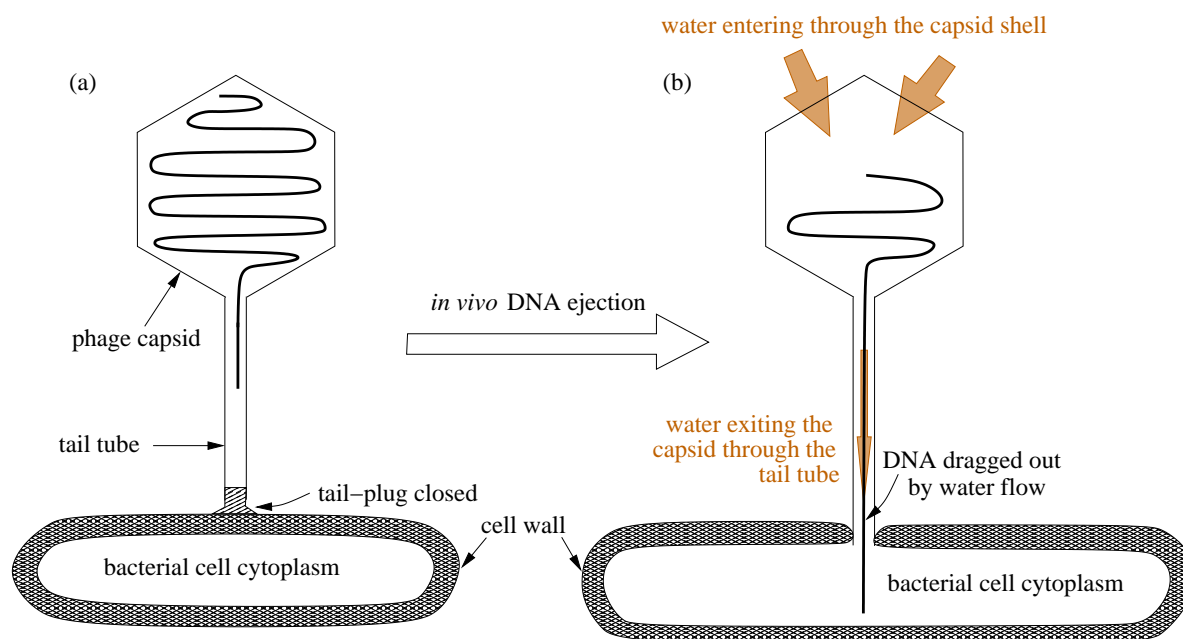
Advocates of  $P_{\text{ejection}}$ -based genome ejection *in vivo* have made various *ad hoc* suggestions to explain how an entire phage genome can enter the cell cytoplasm, including the involvement of cytoplasmic, non-sequence-specific, DNA-binding proteins or condensation of the phage DNA in the crowded cell cytoplasm. There is however, no direct experimental support for any of these ideas. Condensing the entering phage genome in the bacterial cytoplasm would effectively prevent its transcription, but efficient — and immediate — gene expression is precisely the primary strategy of phage infections. Furthermore, with the specific exception of second-step transfer of T5 DNA, it is hard to imagine how entering phage DNA could effectively compete with the 100-fold higher DNA concentration of the bacterial chromosome for those proteins in order to complete genome internalization in a kinetically reasonable time frame. T5 second-step transfer requires the prior synthesis of two T5-encoded DNA-binding proteins, and early T5-encoded nucleases completely degrade the bacterial chromosome [80], thereby removing competitor DNA. Nevertheless, sequence-specific DNA-binding proteins, which have no or fewer binding sites on bulk chromosomal DNA, and can therefore effectively bind to incoming phage DNA *in vivo*, have been shown to catalyze genome internalization of phage T7 and its relatives [69], and also of N4 [20].

*4.2.2. The hydrodynamics model is consistent with the physiology of phage infection* DNA ejection *in vivo* and *in vitro* are actually quite different. The major distinction is that when the tail is unplugged *in vitro*, the environment at the distal end of the tail tube is the same buffer that surrounds the capsid. In contrast, during a natural infection the distal end of the tail tube is in the cytoplasm of the infected cell, while the environment of the capsid is the growth medium. Consequently, the osmotic pressure of the medium that surrounds the phage capsid is different from that at the opening of the tail tip.

We denote the osmotic pressure of the culture medium by  $\pi_{\text{out}}$ , and that of the cytoplasm of the infected cell by  $\pi_{\text{cyt}}$ ; note that  $\pi_{\text{cyt}} > \pi_{\text{out}}$  as the bacteria need to maintain positive turgor in order to grow [77]. Although in  $P_{\text{ejection}}$ -based model of phage genome ejection turgor is assumed to provide a resisting force against  $P_{\text{ejection}}$ , it is the bacterial Achilles heel that actually promotes complete genome ejection by a hydrodynamics mechanism. When the tail plug is removed by interaction with its receptor, water will flow up the osmotic gradient — one that exists between the growth medium and the cell cytoplasm — from the growth medium, through the capsid shell and tail tube into the cell cytoplasm. This directional water flow can drag the DNA into the infected cell. This mechanism is schematically shown in Fig. 6. It should be noted that bacterial cells respond immediately to changes in  $\pi_{\text{cyt}}$  and they will always

maintain some turgor even during phage infection.

When bacteria enter stationary phase, their turgor pressure drops considerably. This reduces the osmotic gradient between the external medium and the cytoplasm, in turn this reduces the strength of water flow from the culture medium into the cytoplasm. This may explain why bacteria in stationary phase are not easily infected by most phages. Notably, T7 is an exception, infecting stationary (and starved) cells at normal efficiency [94], even though subsequent phage development may be compromised by a lack of biosynthetic capacity in the cell.



**Figure 6.** Schematic of DNA ejection *in vivo*. (a) Mature phage adsorption on a bacterial cell. (b) The plug blocking the tail is opened or removed as the cell envelope is breached. Water and small solutes enter the phage capsid from the culture medium and drains through the tail tube into the cytoplasm of the infected cell, dragging the DNA along with it.

Following infection by all but one of the phages we are aware of that have been tested, there is a transient drop in membrane potential and a transient release of intracytoplasmic ions into the external medium [81,82]. Both occur during the period that DNA is thought to be ejected from the virion into the cell cytoplasm. In order to evaluate the role of the membrane potential during T4 infection, an extensive series of electrochemical studies that monitored ion fluxes were conducted (Reviewed in Refs. [83,84]). Experiments were often combined with infective center assays after Hershey-Chase blending of phage-cell complexes, allowing estimates of  $10^3$ - $10^4$  bp/sec for the rate of T4 genome internalization *in vivo* [81,83-85]. Furthermore, T5 releases its DNA in two distinct steps [80]. During each step, cytoplasmic  $K^+$  leaks into the external medium, while during the pause between the steps no leakage occurs [86]. Thus, ion leakage is associated with phage DNA ejection, and if ions are leaking out, water should



be flowing from the external medium through the phage virion into the cell cytoplasm to reduce the osmotic gradient. This is exactly what is expected if hydrodynamic forces are dragging the phage genome into the cell.

There are conflicting published data on membrane depolarization and ion leakage for phage T7, but it is now clear that T7 infection does not result in either phenomenon [87, 88], indicating that no open channel that could allow water flow exists between the environment and the infected cell cytoplasm. These are significant observations, because, unlike other phages, the internalization of T7 and related phage genomes is entirely catalyzed by molecular motors. These motors are enzymes that utilize cellular energy to transport the entire genome into the cell, each functioning at a constant rate regardless of how much DNA remains in the phage head [56, 89–93]. Thus, in the absence of hydrodynamic forces, energy-requiring enzymes are necessary to effect phage genome internalization by the cell.

The hydrodynamic model of DNA ejection does not require secondary *ad hoc* mechanisms to complete genome transfer into the cell. Because the water flow is determined by the values of  $\pi_{\text{cyt}}$  and  $\pi_{\text{out}}$ , the ejection process is (relatively) independent of DNA condensing agents. A bacterial cell responds immediately to changes in  $\pi_{\text{cyt}}$  and will maintain some turgor even during phage infection. Thus, hydrodynamic flow from the external environment through the phage particle into the cell cytoplasm will continue until genome internalization is complete and the channel is closed. (It is not known how this occurs in any phage system, but all models of phage DNA ejection must invoke this step in order to prevent a permanent loss of the cellular membrane potential. If the potential is totally collapsed, the cell would die and no phage progeny would ever result.) Furthermore, although low concentrations of DNA condensing agents have a major effect on  $\pi_{\text{in}}$ , they have little effect on either  $\pi_{\text{out}}$  or  $\pi_{\text{cyt}}$  (e.g., intracellular levels of polyamines, which are critical for ribosome and chromosome stability are tightly regulated). This means that phage DNA ejection *in vivo* should be relatively independent of the presence of DNA condensing agents in the growth medium. Indeed, ongoing experiments in the laboratory of one author (IJM) suggest that the latent periods and burst sizes of various phages (including parallel experiments with  $\lambda$  and the deletion mutant  $\lambda\text{b221}$ , the latter having a substantially reduced value, relative to wild-type  $\lambda$ , of  $\pi_{\text{in}}$ ) are not affected by the presence of 1 mM spermine in the bacterial growth medium.

The hydrodynamic model also provides a mechanism for the ejection of single-stranded genomes and proteins into the cell during infection. This is important as all phages eject protein molecules into the infected cell, and a significant fraction of phages do not contain dsDNA packaged at the high density of  $\sim 500$  mg/ml. Any molecule in the path of water flowing along the osmotic gradient between the external medium and the cell cytoplasm can be driven down the tail tube and into the cell. As this water flow is necessarily vectorial, nucleic acids or proteins to be ejected do not need to be positioned in the tail tube in the mature phage particle. Neither do they need to possess internal energy to drive their ejection from the capsid.

In summary, with the caveat that, to explain the multi-step process of T5

and perhaps other phages ( $\phi 29$  is one known example [95]), an additional *ad hoc* mechanism of protein conformational changes that temporarily block water flow from the external fluid into the cell cytoplasm, in turn temporarily stopping DNA ejection, the hydrodynamic model of phage DNA ejection is consistent with — and importantly can explain — all the observations we are aware of that have been made using any phage-host combination *in vivo*.

However, theories need to be tested experimentally. Determining the kinetics of phage genome internalization into infected cells would go a long way to supporting or refuting competing models. The internal capsid pressure models predict that the late stages of genome ejection should slow towards a zero rate as the residual pressure equals that in the cell cytoplasm, and that a secondary process using, for example, polyamines or DNA-binding proteins to interact with the entering the DNA completes the process. A secondary process is likely to occur with different kinetic parameters and may be experimentally detectable. Conversely, the hydrodynamic model would predict a largely constant rate of genome internalization, as the osmotic pressure gradient between the cell cytoplasm and the environment is unlikely to change substantially while viral DNA enters the cell. Unfortunately, it has proved very hard to measure directly the *in vivo* kinetics of genome entry outside the T7 family of phages, although some measurements have been made with phage N4 [96], and experiments with both  $\lambda$  and T5 are being initiated in the Molineux laboratory. However, it may be possible, using a combination of polyamines and external osmolytes, to increase the cytoplasmic osmotic pressure above that inside the mature phage head. If phage infection still occurs normally under those conditions, it would then provide irrefutable evidence that internal capsid pressure is not important for infection, although it would not provide any direct evidence for the hydrodynamic model.

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